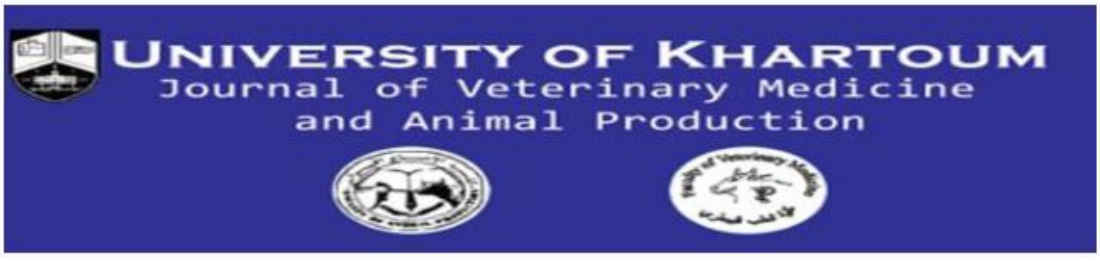
U. of K. J. Vet. Med. Anim. Prod., Vol.8, Issue 2 (2017) p 99 -105
ISSN: 243456



Immunization of Sheep Against Caseous Lymphadenitis

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Abstract

The aim of this study was to evaluate the efficacy of various vaccines against caseous lymphadenitis in sheep. A culture of a local strain of *Corynebacterium pseudotuberculosis* CP41 N was produced using IBT bioreactor at pH 8, temperature 31 º C and dilution rate of 0.02 h^{-1}. The harvest was used to prepare five types of vaccines; The first was a killed whole cell vaccine containing 8mg bacterial dry weight / 0.5ml, the second was a cell wall vaccine containing 3mg protein/ 0.5 ml, the third was a toxoid vaccine containing 4 mg protein /0.5 ml, the fourth was the cell wall + the toxoid vaccine (3mg protein/ 0.5ml+ 4mg protein /0.5ml) and the fifth was the killed whole cell + the toxoid vaccine (8mg bacterial dry weight /0.5ml + 4 mg protein/0.5ml). Saponin and aluminum hydroxide adjuvants were added in a concentration of 1mg/1ml dose each to the different vaccines. Each vaccine was injected subcutaneously in animals of one of 5 groups of sheep of 7 animals each, and animals in the sixth group were left as unvaccinated control. All animals were bled at weekly intervals for serological monitoring by the agglutination test. After four weeks, a booster dose of each vaccine as the 1st dose was given to the animals. Four weeks after the second dose of vaccine, all animals were challenged subcutaneously with 5.1X108 CFU of *C. pseudotuberculosis*CP41 N. The animals were then slaughtered four weeks later to determine the number of abscesses in the carcasses. The results showed that the vaccine which consisted of killed whole cells + the toxoid was the best of the five vaccines with a significant reduction ($p<0.05$) in mean abscess numbers in vaccinated animals after challenge with *C. pseudotuberculosis*. The percentage of protection of the group vaccinated with the killed whole cell+ the toxoid was 75% compared with 32.3%, 37.5%, 37.5% and 45.3% for the groups vaccinated with the cell wall lysate, the killed whole cell, the toxoid and the cell wall lysate + the toxoid vaccines, respectively.

Key words: *Sheep, caseous lymphadenitis, Corynebacterium pseudotuberculosis*

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المستخلص

هدفت هذه الدراسة لتقييم فعالية لقاحات مختلفة ضد مرض التهاب العقد اللمفية الجبنى أو السل الكاذب فى الضأن. انتجت خلايا مركزة من سلالة محلية لبكتريا وتدية السل الكاذب التى تسبب المرض باستخدام جهاز المفاعل الحيوي لأول مرة داخل السودان فى أس هيدروجينى (pH 8) ودرجة حرارة (31 ºC) ومعدل تخفيف 0.02 h^{-1}). من هذا المنتج تم تجهيز خمسة أنواع من اللقاحات كالآتى: الأول لقاح الخلية الكاملة الميت ويحتوى على 8 مج من الوزن الجاف /0.5 مل)، الثانى لقاح حلالة جدار الخلية و يحتوى على3 مج بروتين /0.5 مل ، الثالث لقاح الذوفان ويحتوى على 4 مج بروتين /0.5 مل ، الرابع لقاح حلاله جدار الخلية مضافا إليه الذوفان ويحتوى على(3 مج بروتين / 0.5 مل + 4 مج بروتين / 0.5 مل) والخامس لقاح الخلية الكاملة الميت مضافا إليه الذوفان (8 مج وزن جاف/0.5 مل +4 مج بروتين/0.5 مل) . تم حقن جرعتين بفارق أربع أسابع بين الجرعة الأولى والثانية من هذه اللقاحات في كل مجموعة من خمس مجموعات من الضأن تحتوى كل واحدة منها على سبع حيوانات وتركت مجموعة سادسة من غير حقن للضبط. جمعت عينات دم للأمصال من كل حيوان إسبوعيا لمعرفة الاستجابة المناعية بقياس مستوى الأجسام المضادة باستخدام اختبار التراص البكتيرى. بعد أربع أسابيع من الجرعة الثانية تم حقن كل الضأن تحت الجلد بجرعة تحدى موحدة من نفس السلالة وبعد أربع أسابيع من التحدي ذبحت كل الحيوانات و تم فحصها بحثا عن آفات المرض وتحليل النتائج إحصائياً . بعد تقييم درجة الحماية التي أحدثتها هذه اللقاحات في الحيوانات بعدالتحدى، كان افضل لقاح المكون من الخلية الكاملة مضافا اليها الذوفان، وكان الفرق معنويا ($P<0.05$) فى تقليل متوسط عدد الآفات مقارنة ببقية اللقاحات ، وكانت نسبة الحماية منانتشار الآفات في الحيوانات التي حقنت بهذا اللقاح 75% مقارنة بـ 32.3%، 37.5%، 37.5% و 45.3% فى الحيوانات التي تم حقنها بلقاح حلالة الخلية، لقاح الخلية الكاملة، لقاح الذوفان ، ولقاح حلالة الخلية زائداً الذوفان على التوالى .

كلما ت مفتاحية :*الضأن، التهاب العقد اللمفية الجبنى، بكتريا وتدية السل الكاذب*

Introduction

Caseous lymphadenitis causes great economical losses in sheep and goats' production due to condemnation of animals or parts of carcasses because of the infection (Williamson, 2001; Paton, 2003). It affects animal trade and detection of the external disease in few animals leads to rejection of a whole export shipment as internal lesions may not be detected till after slaughter. Surgical treatment of the disease is not successful as it may recur and it causes extensive fibrosis at the site of infection. Moreover, lancing and draining the superficial abscesses contaminate the skin and the surrounding environment. The unguided use of antibiotics increases the problem of antibiotic resistance. Thus, to combat the disease efforts should be directed to produce a protective vaccine against the disease. Several vaccines were developed using different antigens, but they all showed variable results and none of them was reliable in the control the disease in sheep and goats.

The aim of this study was to produce various vaccines from a mass culture of *C. pseudotuberculosis* using IBT bioreactor and to determine their efficacy in controlling caseous lymphadenitis (CLA) in sheep

Materials and methods

Mass cultivation of *C. pseudotuberculosis* using IBT Bioreactor

A local strain of *C. pseudotuberculosis* strain CP 41N isolated from a sheep with CLA was used as a seed for inoculation in the bioreactor (BBC, Germany). This strain was selected because of its high growth yield and increased haemolytictitre (Abdel

Wahab, 2000). The lyophilized strain was revitalized by s/c injection into a rabbit, after it died or an abscess appeared at the injection site, the rabbit was autopsied and material from abscess was then transferred to brain heart infusion broth with 0.1% Tween 80 (BHIB+T). One milliliter of the broth was inoculated into 50 ml of BHIB+T and incubated at 37 ºC for 24 hours. Growth could be detected by turbidity and smears were made from the broth, and stained by Gram's stain to check for purity. This 50 ml inoculum was used for inoculating the bioreactor containing Burrell's liquid media (Burrell, 1979).

The bioreactor parts were assembled, autoclaved, a temperature of 31º C, pH of 8.0 and a dilution rate at 0.02 h^{-1} were adjusted (Suleiman, 2001). The volume of the media in the system was 500 ml. The system was circulated after inoculation, samples were taken daily and smears were stained with Gram's stain to check purity. The viable cell count of *C. pseudotuberculosis*in samples from the bioreactor as determined according to Miles *et al.*, (1938), was 1.5 x 108 CFU/ml. The harvested bioreactor broth was collected in 0.3% formal saline (Bőhnel, 1999). For separation of cells and toxins, polypropylene hallow fiber filters (FRESENIUS, St. Augustin) SPS900 (MWCO/1000 kDa) and SPS600 (MWCO/100 kDa) were used. Two phases of filtration were done to obtain purified and concentrated products from the formalinized bioreactor broth, which could be carried out either simultaneously or separately.

Preparation of vaccines

Five vaccines prepared were: a killed whole cell, a cell wall lysate, a toxoid, a killed whole cell + toxoid and a cell wall lysate + toxoid.

(i) The killed whole cell concentrate was prepared after collection and filtration of harvested formal saline solution. The separated cells were washed three times with distilled water and stored at 4º C. The 0.5ml dose of the killed whole cells, the 1st vaccine was consisted of 8 mg of bacterial dry weight.

(ii) To prepare the cell wall lysate, the killed whole cell concentrate was washed twice with acetone and twice with diethyl ether. The cells were then disrupted either by shaking with glass beads by means of an electronic shaker (Edmond, Germany) and/ or by sonicator (MSE, England). Trypsin 1ug/ml was added for digestion of protoplasm and the suspension was incubated at 37º C for six hours. By centrifugation, the cell wall lysate was pelleted, and then washed three times in distilled water. The lysate was examined microscopically after staining with Gram's stain to detect intact cells; the procedure was repeated whenever intact cells were observed in the smears. The protein content was estimated according to Lowry *et al.*, (1951) method which depends on detection of tyrosine content that is constant in many proteins. The 0.5ml dose of the cell wall lysate, the 2nd vaccine was consisted of 3mg of protein.

(iii) The toxoid vaccine was prepared by filtration of the formalinized bioreactor culture harvest after cells separation. The concentration of formalin was maintained at 0.3%, and then incubated at 37º C for 45 days, then the toxoid was stored at 4º C. The

0.5 ml dose of the toxoid, the 3rd vaccine consisted of 4mg of protein.
(iv)The 1ml dose of the killed whole cells+ toxoid, the 4th vaccine composed of 0.5 ml/8mg bacterial dry weight of killed whole cells and 0.5ml/4mg protein of the toxoid.
(v) The 1ml dose of the cell wall lysate + toxoid, the 5th vaccine composed of 0.5ml/3mg protein of cell wall lysate and 0.5ml/4mg protein of the toxoid.
Saponin and aluminum hydroxide Al $(OH)_3$ were incorporated into each vaccine at a dose rate of 1mg/1ml each.

Vaccination trials

Forty two sheep of local types at different ages from six months to one year old, weighing 15-30kg and seronegative for *C. pseudotuberculosis* by the bacterial agglutination test were bought from the local market. The animals were divided at random into 6 groups, 7 sheep each. They were kept in pens, and dewormed. Feed and water were provided *ad libitum* and observed for adverse clinical signs for five weeks before vaccination.

Animal in 5 groups were given the first dose subcutaneously in the shoulder region. After 4 weeks a booster dose of each vaccine was injected similarly into each animal. Animals in in the 6th group were kept as non-vaccinated controls.

A challenge dose of 5.1X108 CFU of *C. pseudotuberculosis* strain CP41 N was determined according to Miles *et al.,* (1938). Four weeks after the booster dose of each vaccine, all sheep in the 6 groups were challenged subcutaneously on the left side of the neck of each animal with the challenge dose.

Serology

Serological responses to the vaccines were monitored by the bacterial agglutination test. The antigen for the test was prepared by culturing *C. pseudotuberculosis* strain CP41N onto brain heart infusion (BHI) agar containing 0.1% sorbitan monooleate at 37°C. The 24- hours growing colonies were transferred to BHI broth containing 0.1% sorbitan monooleate and incubated with constant stirring for six hours at 37°C. The culture was centrifuged at 3000 rpm for 15 minutes. The packed bacterial cells were washed twice with phosphate buffered saline solution (PBSS) at pH 7.2 containing 1.0% sorbitan monooleate and 0.3% formalin, and then suspended in PBSS at the same pH containing 1.0 % sorbitan monooleate (Keskintepe, 1976) and 0.2 % formalin and diluted to 80% transmittance in a spectrophotometer (Milton Roy, U.S.A) at 600 nm wave length (Lund et al., 1982). Double dilutions of each test serum were made from: in 0.5 ml PBSS at pH 7.2 with 0.1% sorbitan monooleate to give 1/5, 1/10, 1/20 to 1/320 dilutions before 0.5 ml of the antigen were added to each tube. The mixture was incubated overnight in a water bath at 37°C and examined visually for agglutination and clearing of the supernatant. A positive control of pooled sera from previously known infected three sheep was used.

Autopsy of sheep after challenge

All animals in the six groups were slaughtered 4 weeks after challenge and examined for abscess formation. As CLA is characterized by abscesses formation in lymph nodes draining the site of infection, the challenge site, the main lymph nodes; prescapular, parotid, mandibular, mediastinal,

mesenteric and supramammary in addition to livers, lungs and subcutaneous tissues of the carcasses were thoroughly examined for pyogenic lesions or abscesses. Samples from all lesions were collected aseptically for bacteriological examinations.

The protective capacity of the vaccines was calculated according to the formula:

% protection= % inf con – % inf vacX100

% infected controls

Statistical analysis

Appearance of abscesses in the control group and groups of animals injected with various vaccines was evaluated statistically using Analysis of variance (ANOVA).

Results

Serological responses to vaccines

Animals vaccinated with killed whole cell, cell wall lysate, cell wall lysate+ toxoid and Killed whole cell+ toxoid showed serological responses by agglutination test. Titres began to increase and reached primary peaks 2 weeks post vaccination, and then declined. Secondary peaks of the titres were observed 1 week after the booster doses. In the four groups of animals vaccinated with killed whole cell, cell wall lysate, cell wall lysate + toxoid and killed whole cell+ toxoid, the agglutination tests showed similar pattern in duration and degree of response. A rise in agglutination titres was evident in the first week following vaccination, and by the end of the second week it began to fall and reached its lowest level by the end of the fourth week (Fig. 1). The titres in sheep vaccinated with the toxoid and non-vaccinated control animals remained unchanged till s/c challenge with virulent *C. pseudotuberculosis* CP41N, when the animals began to respond and the titres reached their peaks 2 weeks after challenge.

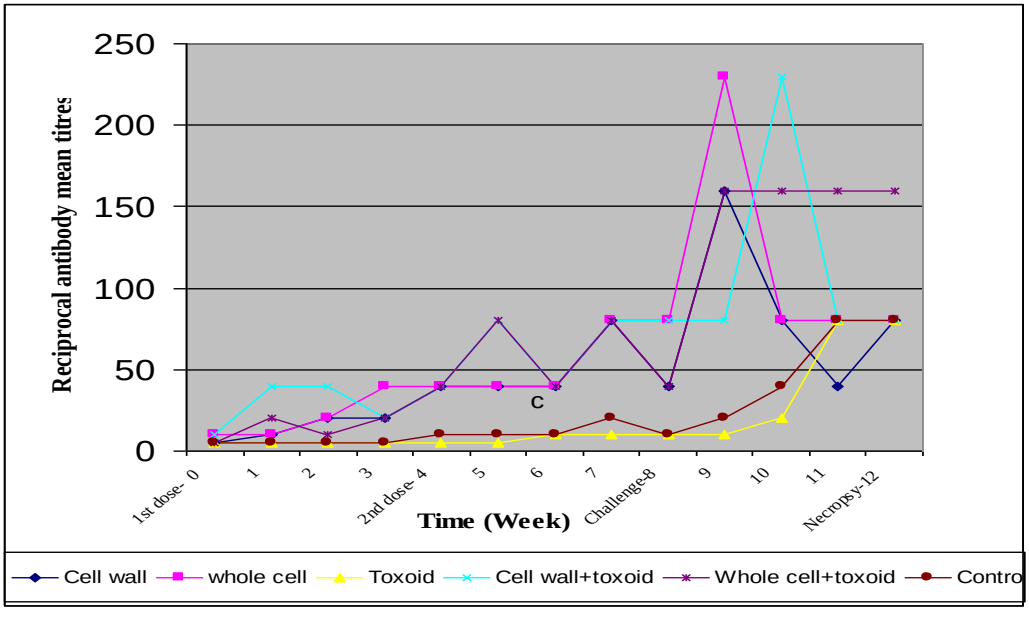

Fig. 1: Agglutination titres of sheep injected with different *C. pseudotuberculosis* vaccines

Response to challenge

Three days after s/c challenge with 5.1X108 CFU/ml of *C. pseudotuberculosis* strain CP41N, 3 animals in the control group died. There were no abscesses at the sites of challenge or internal organs, but there were acute inflammation and distinct enlargement of the prescapular lymph nodes draining the site of challenge (Fig. 2).

Six of the vaccinated animals died from pneumonia before challenge, but there were no deaths among all vaccinated animals after challenge. Four weeks after challenge, all remaining controls and vaccinated sheep were slaughtered. The distribution of abscesses in animals were recorded and statistically analysed. Pus from s/c all lesions were positive for *C. pseudotuberculosis* upon culture.

Protection due to the different vaccines was found to be 37.5%, 32.3%, 37.5%, 45.3% and 75% in animals vaccinated with killed whole cell, cell wall lysate, toxoid, cell wall lysate +toxoid and killed whole cell + toxoid, respectively (Table 1).

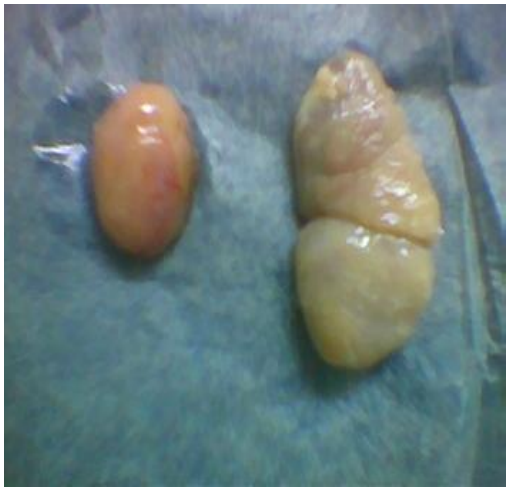

Fig. 2: Presacpular lymph nodes of a control sheep that had died after challenge with C. *pseudotuberculosis*. The lymph node on the right is the one draining the challenge injection site and the left is the one draining the other side.

When analysis of variance (ANOVA) was used to evaluate and compare the response to challenge in vaccinated and control animals. The results showed that the mean difference between groups was significant ($P<0.017$). The mean difference was significant ($P<0.05$) when the group of animals vaccinated with killed whole cell +toxoid was compared with control group and group of animals vaccinated with toxoid, and less significant when compared with the group of animals vaccinated with killed whole cell, cell wall lysate, and cell wall lysate +toxoid, probably due to the small

sample size as animals that died before challenge had to be excluded.

Table 1: Protection of vaccinated animals

Animal group	No. of organs	No. of infected organs	Infected organs (%)	Protection (%)
G1	48	13	27.1	32.3
G2	32	8	25	37.5
G3	16	4	25	37.5
G4	32	7	21.9	45.3
G5	40	5	10	75
G6	40	16	40	0

G1 (CW): Group 1, cell wall vaccinated animals, **G2** (WC): Group 2, whole cell vaccinated animals, **G3** (T): Group 3, toxoid vaccinated animals, **G4** (CW+T): Group 4, cell wall+ toxoid vaccinated animals, **G5** (WC+T): Group 5, whole cell+ toxoid vaccinated animals, **G6**, Group 6, Control

Discussion

Confluent growth culture of *C. pseudotuberculosis* culture was successfully obtained using IBT bioreactor for the first time in Sudan, which showed the possibility of mass production of a good quality culture in a short time. Five types of vaccines were tried to immunize sheep against CLA. The results showed that killed whole cell + toxoid was the most protective among these vaccines. A significant reduction in abscess formation was noticed after challenge with *C. pseudotuberculosis* in sheep vaccinated with this vaccine compared with unvaccinated control animals. The protection percentage for the group vaccinated with this vaccine reached 75% compared with 37.5%, 32.3%, 37.5% and 45.3% for groups vaccinated with killed whole cell, cell wall lysate, toxoid and cell wall lysate + toxoid respectively. These results in agreement with the findings of Simon-Valencia, (1992) who reported that defatted killed whole cell of *C. pseudotuberculosis*with toxoid gave better, but not complete protection to challenged mice. However, Eggleton *et al.* (1991a) postulated that the addition of whole cells did not improve the protective potency of *C. pseudotuberculosis* toxoid vaccines. It was argued that their vaccine, which had been prepared from crude toxoid could contain soluble antigens other than exotoxin, and they did not preclude the possibility that more than one antigen was responsible for the protection that had been conferred.

In a previous study it was found that cell wall lysate + toxoid produced a protection percentage of 65% in goats (Abdel Wahab 2000), this rate is higher than the one observed in the present study in sheep (45.3%) using the same type of vaccine possibly due to species variation in the immunological response.

Sheep vaccinated with toxoid alone were as susceptible to infection as the control animals. Five of seven animals in this group died before challenge, i.e. only two animals remained which made statistical evaluation of vaccination in this group unreliable.

The challenge dose of 5.1X108 CFU/ml of *C. pseudotuberculosis* inoculated subcutaneously was higher than natural infection dose with *C. pseudotuberculosis* through the skin. *C. pseudotuberculosis* may be transmitted to sheep by contact of skin abrasions with contaminated soil or fomites (Hein and Cargill, 1981). A gram of pus may contain 106 to 107 CFU/ml of *C. pseudotuberculosis* (Augustine *et al.* 1982); however, as this pus becomes dried and dispersed in soil or on fomites, the concentration would decrease. Therefore, a natural challenge dose would be less than 5.1X108 CFU/ ml of *C. pseudotuberculosis*.

Death of control animals after challenge due to acute toxicity was reported by Nairn *et al.* (1977). The results of this study confirm this observation as 3 animals in the control group died within 24 hours after challenge. The development of suppurative lesions at the site of challenge in control and vaccinated animals with varied degrees was reported by Nairn *et al.* (1977) and our findings are in agreement with this.

Bacterial agglutination test was used for monitoring the humoral response in vaccinated then challenged animals. The results obtained were similar to those of Shigidi, (1979).

Conclusion

It could be concluded that the vaccine consisting of killed whole cell+ toxoid appears to be promising for protection against the disease, and trials of this vaccine under field conditions are recommended.

Acknowledgement

The authors would like to thank technician S. A. Nogaand A. Abdalla for their technical assistance. The permission of the Director, General of the Animal Resources Research Corporation is acknowledged.

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